

Role of Leaky Neuronal Ryanodine Receptors in Stress-Induced Cognitive Dysfunction

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SUMMARY

The type 2 ryanodine receptor/calcium release channel (RyR2), required for excitation-contraction coupling in the heart, is abundant in the brain. Chronic stress induces catecholamine biosynthesis and release, stimulating β -adrenergic receptors and activating cAMP signaling pathways in neurons. In a murine chronic restraint stress model, neuronal RyR2 were phosphorylated by protein kinase A (PKA), oxidized, and nitrosylated, resulting in depletion of the stabilizing subunit calstabin2 (FKBP12.6) from the channel complex and intracellular calcium leak. Stress-induced cognitive dysfunction, including deficits in learning and memory, and reduced long-term potentiation (LTP) at the hippocampal CA3-CA1 connection were rescued by oral administration of S107, a compound developed in our laboratory that stabilizes RyR2-calstabin2 interaction, or by genetic ablation of the RyR2 PKA phosphorylation site at serine 2808. Thus, neuronal RyR2 remodeling contributes to stress-induced cognitive dysfunction. Leaky RyR2 could be a therapeutic target for treatment of stress-induced cognitive dysfunction.

INTRODUCTION

Acute stress enhances cognitive function via activation of the sympathetic nervous system as part of the “fight-or-flight” response (McEwen and Sapolsky, 1995). In contrast, chronic stress causes cognitive dysfunction associated with glucocorticoid-induced hippocampal neuron damage (McEwen and Sapolsky, 1995). However, the precise mechanisms underlying stress-induced cognitive dysfunction remain poorly understood, and there is currently no effective therapy.

Intracellular calcium (Ca^{2+}) homeostasis plays a crucial role in neuron survival and function. Neuronal Ca^{2+} influx is mediated through voltage-gated channels and/or receptor-operated channels activated by neurotransmitters. Intracellular Ca^{2+} re-

lease occurs via ryanodine receptors (RyRs) and inositol (1,4,5)-trisphosphate receptors (IP3Rs) on the endoplasmic reticulum (ER) (Berridge et al., 2003). There are three isoforms of RyR in the brain. RyR1 and RyR2 are both expressed in the hippocampus. RyR2 expression is upregulated in rat hippocampus following intensive training tasks (Cavallaro et al., 1997; Zhao et al., 2000). RyR2 knockdown impairs performance of mice in passive avoidance tests (Galeotti et al., 2008), whereas RyR1 knockdown did not (Galeotti et al., 2008).

Ca^{2+} released into the cytoplasm is pumped back into the ER by the ER Ca-ATPase (SERCA) or removed from the cell by the plasma membrane Ca-ATPase (PMCA) and $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Altered Ca^{2+} homeostasis contributes to neuronal degeneration and cell death (Mattson, 2007) and defective post-synaptic signaling, presynaptic control of neurotransmitter release, and long-term synaptic plasticity (Berridge, 1998; Fitzjohn and Collingridge, 2002).

We have shown that binding of the subunit calstabin2 (FKBP12.6) to RyR2 channels in the heart is impaired under stress conditions by protein kinase A (PKA) phosphorylation at serine 2808 (Wehrens et al., 2005) and oxidation/nitrosylation of the channel such that calstabin2-depleted RyR2 channels leak intracellular Ca^{2+} (Shan et al., 2010a, 2010b). A novel class of drugs (derivatives of 1,4-benzothiazepines), known as intracellular calcium release channel stabilizers, or Rycals (e.g., S107 and JTV-519) (Bellinger et al., 2008b), prevents stress-induced depletion of calstabin from RyR and fixes the pathological intracellular Ca^{2+} leak. Rycals inhibit heart failure progression in mice (Shan et al., 2010a) and prevent catecholamine-induced arrhythmias (Lehnart et al., 2008; Shan et al., 2010a; Wehrens et al., 2004; Yano et al., 2003).

We now show that stress-induced oxidation, nitrosylation, and PKA hyperphosphorylation deplete calstabin2 from brain RyR2, resulting in leaky channels that contribute to stress-induced cognitive dysfunction in mice. The orally available small molecule S107 decreased ER Ca^{2+} leak via neuronal RyR2 and prevented stress-induced cognitive dysfunction. Genetic ablation of the RyR2 PKA phosphorylation site in *RyR2-S2808A^{+/+}* mice (Shan et al., 2010b; Wehrens et al., 2005) also protects against stress-induced cognitive dysfunction. Taken together, these results establish a crucial role for leaky neuronal RyR2 in

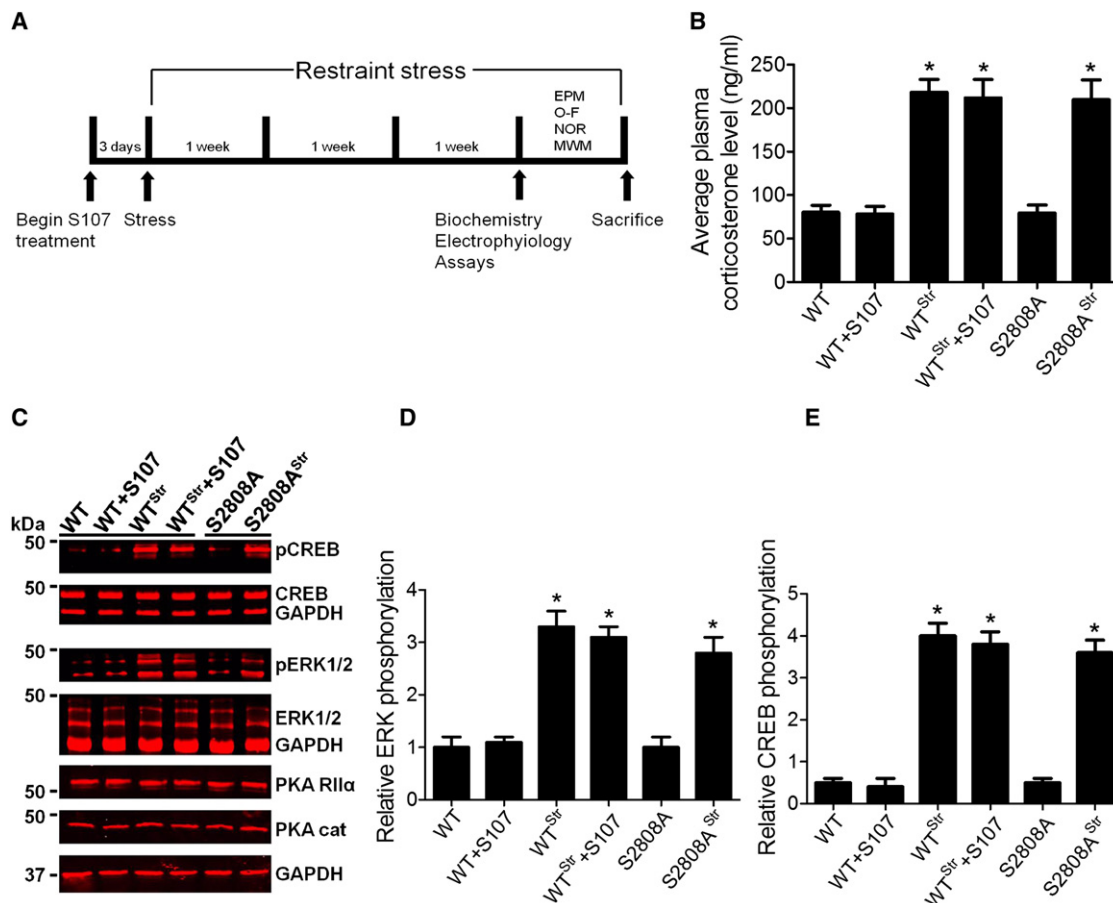


Figure 1. Chronic Stress Response in WT and in the *RyR2-S2808A*^{+/+} Mice

(A) Mice were randomly divided into groups as indicated. Stressed mice were subjected to chronic restraint stress in Plexiglas restraint tubes overnight daily for 3 weeks. S107 (75 mg/kg/day) in drinking water was begun 3 days before stress and throughout the experiment.

(B) Plasma corticosterone levels were assayed in the morning. Data are mean \pm SEM, $n = 4$.

(C) Representative immunoblots showing pCREB and pERK in control and stressed mice. Hippocampal samples were isolated immediately after 3 weeks of stress from WT and *RyR2-S2808A*^{+/+} mice.

(D and E) Summary data (mean \pm SEM, $n = 5$) are shown for pCREB (C) and pERK (D). Total CREB, total ERK, and GAPDH are loading controls.

See also Figure S1.

stress-induced cognitive dysfunction and suggest a potential therapeutic approach to preventing stress-induced cognitive dysfunction and posttraumatic stress disorder (PTSD).

RESULTS

Hippocampal Stress Response

Wild-type (WT) and *RyR2-S2808A*^{+/+} mice were subjected to chronic restraint stress (Copeland et al., 2005; Fukui et al., 1997; Patel et al., 2004) with or without treatment with the Rycal S107 in the drinking water (Figure 1A). There was a >2-fold sustained increase in plasma corticosterone (CORT) in WT and *RyR2-S2808A*^{+/+} mice following 3 weeks of chronic restraint stress (Aguilera, 1994; Carrasco and Van de Kar, 2003; McEwen, 2004, 2007) (Figure 1B and Figure S1C available online). The immediate early gene c-Fos was significantly elevated after acute stress and, to a lesser degree, after acute stress following

chronic stress, indicating some blunting of the hypothalamic pituitary axis (HPA) (Figures S1D–S1F) that was consistent with previous reports (Perrotti et al., 2004). The transcription factors CREB and ERK regulate tyrosine hydroxylase and catecholamine biosynthesis and are phosphorylated under stress conditions (Brazelton et al., 2000; Impey et al., 1998; Liu et al., 2005, 2008; Sabban et al., 2004; Shaywitz and Greenberg, 1999). There was a significant increase in CREB (pCREB) and ERK (pERK) phosphorylation in hippocampal, cortical, and whole brain from stressed mice, which is consistent with a general stress response in the brain (Figures 1C–1E and S2) and increased PKA activity (Figure S2G). PKA catalytic or regulatory subunits were unchanged (Figure 1E).

CRF, ACTH, and CORT were significantly increased at 15 min after termination of chronic stress in all stressed groups (Figures S1A–S1C). CRF and ACTH fell to control levels 1 hr postchronic stress (Figures S1A and S1B); however, CORT levels remained

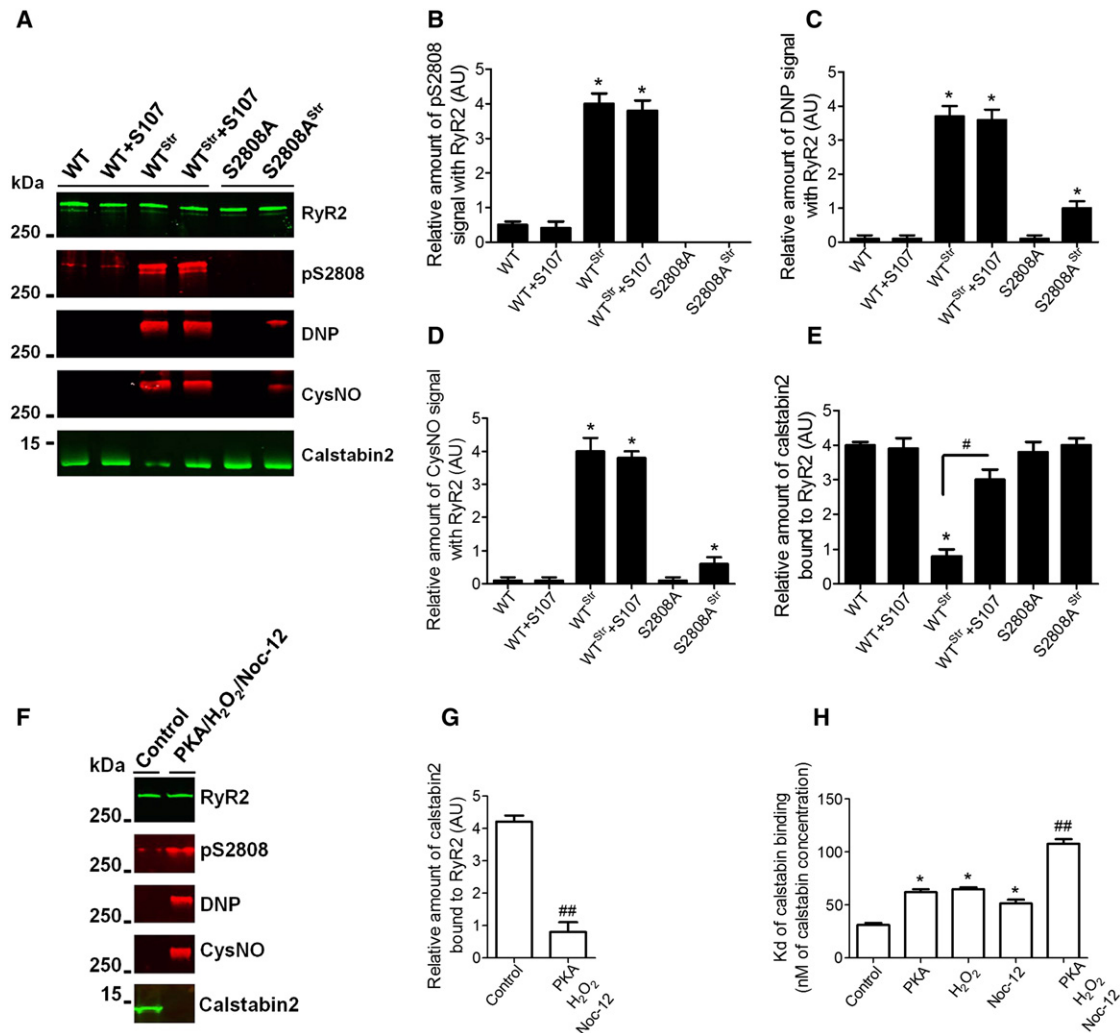


Figure 2. Stress-Induced Remodeling of Neuronal RyR2 Macromolecular Complexes

(A) Hippocampal RyR2 was immunoprecipitated and immunoblotted to detect PKA hyperphosphorylation, oxidation, and Cys S-nitrosylation of RyR2 and calstabin2 as previously described (Marx et al., 2000; Reiken et al., 2003b; Shan et al., 2010a, 2010b; Ward et al., 2003).

(B–E) Summary data for RyR2-pS2808, Cys S-nitrosylation, DNP (oxidation), and calstabin2. Data are mean \pm SEM, * p < 0.05, # p < 0.05, n = 5. The mean for the unstressed control was set = 1.0.

(F) ER microsomes were resuspended and treated with PKA (40 units), H₂O₂ (1 mM), and Noc-12 (100 μ M) separately or in combination. Treated microsomes were immunoprecipitated and immunoblotted with the antibodies described in (A)–(E).

(G) Summary data for F; immunoblot data for calstabin2/RyR2.

(H) Kds for ³⁵S-calstabin2 binding to RyR2.

Data are mean \pm SEM, * p < 0.05, # p < 0.05, ## p < 0.01, n = 5. See also Figures S3, S4, and S5.

significantly elevated at 24 hr poststress, indicating a persistent stress response (Figure S1C). There was a significant but blunted elevation of CRF, ACTH, and CORT after acute stress in chronically stressed WT and RyR2-S2808A^{+/+} mice (Figures S1A–S1C), indicating that the HPA axis was intact but blunted (Figures S1A–S1C).

Stress-Induced Remodeling of the RyR2 Macromolecular Complex

Neuronal RyR2 were PKA hyperphosphorylated, oxidized, S-nitrosylated, and depleted of calstabin2 in hippocampal and

whole brain (Figures 2A–2E and S3A–S3E). Neuronal ER SERCA activity was unchanged in stressed mice (Figure S3F). S107 given in drinking water (75 mg/kg/d) prior to and during the stress protocol prevented calstabin2 depletion from the RyR2 macromolecular complex without altering PKA phosphorylation, oxidation, or S-nitrosylation of RyR2 (Figure 2E). S107 improves cardiac and skeletal muscle function (Anderson et al., 2011; Bellinger et al., 2009; Shan et al., 2010a, 2010b). To show that the effects of S107 in the present study were due to its neural as opposed to extraneural actions, mice were treated with a related Rycal (S36, 75 mg/kg/day)

that also improves cardiac and skeletal muscle function but does not cross the blood-brain barrier (BBB). In contrast to S107, S36 did not prevent calstabin2 depletion from neuronal RyR2 in hippocampal samples from stressed mice (Figures S4A–S4E).

PKA, H_2O_2 , and Noc-12 individually caused an ~2-fold decrease in the binding affinity of calstabin2 to RyR2 ($p < 0.05$) (Figures 2F–2H, S5A, and S5B) and, in combination, caused an ~4-fold decrease in the binding affinity of calstabin2 to RyR2 ($p < 0.01$) (Figures 2H, S5A, and S5B) as determined by ^{35}S -calstabin2 binding and ~80% depletion of calstabin2 from RyR2 assessed by coimmunoprecipitation ($p < 0.01$) (Figures 2F and 2G). Anti-calstabin2 antibody coimmunoprecipitated RyR2 (Figure S5C). There was no stress-induced change in total neuronal RyR2 or calstabin2 (Figure S5D).

PKA phosphorylation, oxidation, and nitrosylation of RyR2 cause calstabin2 depletion from cardiac RyR2 during heart failure (Lehnart et al., 2004; Shan et al., 2010a; Wehrens et al., 2004). Mice harboring RyR2 channels that cannot be phosphorylated by PKA (*RyR2-S2808A^{+/+}*) are protected against heart failure progression (Shan et al., 2010b; Wehrens et al., 2006). Stress-induced depletion of calstabin2 from hippocampal or whole brain RyR2 was not observed in *RyR2-S2808A^{+/+}* mice (Figures 2A–2E and S3A–S3E). Thus, S107 or genetic ablation of the RyR2 PKA phosphorylation site prevent calstabin2 depletion from neuronal RyR2 in stressed mice.

Leaky Neuronal RyR2 Channels in Chronically Stressed Mice

Single-Channel Activity

The stress-induced remodeling of the cardiac RyR2 macromolecular complex results in “leaky” channels (Lehnart et al., 2008; Shan et al., 2010a; Wehrens et al., 2004). We sought to determine whether stress-induced remodeling of hippocampal RyR2 channels also results in leaky channels with higher single-channel open probabilities (P_o) under resting conditions (e.g., with low-cytosolic-activating [Ca^{2+}]). In WT control mice, the RyR P_o was essentially zero at low cytosolic [Ca^{2+}] (150 nM) (Figures 3A and 3F), whereas RyR channels from stressed mice exhibited a significantly increased P_o (Figures 3B and 3F). RyR channels from S107-treated stressed mice exhibited low P_o , which was similar to nonstressed mice (Figures 3C and 3F). RyR in nonstressed and stressed *RyR2-S2808A^{+/+}* mice showed no increased P_o (Figures 3D–3F). Whole-brain RyR exhibited similar increases in single-channel P_o and normalization when mice were treated with S107 compared to hippocampal RyR channels (Figure S3H).

Intracellular Ca^{2+} Leak

Stress significantly increased ER Ca^{2+} leak ($33.13 \pm 4.2\%$ of uptake in stress, $n = 7$, versus $21.75 \pm 1.82\%$ of uptake in non-stress, $n = 9$, $p < 0.05$ by one-way analysis of variance [ANOVA]) (Figures 3G and 3H). Treatment with S107 in the drinking water prior to and during the stress significantly reduced ER Ca^{2+} leak ($13.51 \pm 1.72\%$ of uptake in S107-treated stress, $n = 7$, versus stress, $p < 0.05$ by one-way ANOVA) (Figures 3G and 3H). There was no significant difference in ER Ca^{2+} leak between stressed ($10.10 \pm 0.67\%$ of uptake, $n = 7$) and nonstressed *RyR2-S2808A^{+/+}* ($10.14 \pm 1.74\%$ of uptake, $n = 7$, $p > 0.05$ by

one-way ANOVA) or between WT and *RyR2-S2808A^{+/+}* mice (Figures 3G and 3H).

Thus, chronic stress-induced remodeling of neuronal RyR2 channels caused increased single-channel P_o under resting conditions and ER Ca^{2+} leak that was prevented by treatment with S107 or genetic ablation of the RyR2-S2808 PKA phosphorylation site.

Impaired Cognitive Function and Hippocampal Synaptic Plasticity in Stressed Mice

Mice were examined by using a Morris water maze (MWM) to assess hippocampal-dependent learning and memory (Morris, 1984). WT nonstressed and stressed 3-month-old mice exhibited no differences in swimming velocities, indicating that there was no locomotor defect (Figure S3G). The time spent to reach the hidden platform (escape latency) was prolonged in WT-stressed mice (50.85 ± 3.09 s, $n = 21$) compared to non-stressed control mice (28.39 ± 3.79 s, $n = 21$, $p < 0.05$) at day 3 of the 5 day trial (Figure 4A). Stressed WT mice exhibited prolonged escape latency on days 3–5 ($p < 0.01$), indicating impaired learning due to chronic restraint stress (Figure 4A).

S107 in the drinking water (75 mg/kg/day) significantly reduced escape latency at day 3 (26.61 ± 3.31 s, $n = 22$) compared to the nontreated stressed mice (50.85 ± 3.09 s, $n = 21$, $p < 0.05$) (Figure 4A). On day 6, a probe trial was performed in which the mice attempted to locate the previously hidden platform that had been removed. The percentage of time spent in the target quadrant and the number of target crossings in control nonstressed WT mice ($52.3 \pm 4.8\%$; 4.3 ± 0.59 , $n = 21$) were significantly higher than those in stressed WT mice ($32.50 \pm 2.86\%$, $n = 21$, $p < 0.05$; 1.59 ± 0.36 , $n = 21$, $p < 0.05$) (Figures 4B and 4C), suggesting impaired learning in stressed mice. The stress-induced learning impairment was prevented by S107 treatment as both the time spent in the target quadrant and the number of target crossings were increased in the treated animals ($54.58 \pm 2.56\%$, $n = 22$, $p < 0.05$; 5.67 ± 0.34 , $n = 22$, $p < 0.05$) (Figures 4B and 4C). A lower dose of S107 (50 mg/kg/day) or beginning treatment after the chronic stress did not prevent stress-induced cognitive dysfunction as assessed by using the MWM (Figures S4I–S4K). The Rycal S36 (75 mg/kg/day), which does not cross the BBB, did not improve either the time spent in the target quadrant or the number of target crossings (Figures S4F–S4H).

The novel object recognition (NOR) task was used to assess hippocampal-type memory (Bevins and Besheer, 2006; Denny et al., 2012). There were no significant differences in the total exploration duration among groups in phase 1, the habituation phase, when mice were exposed to two identical objects (Figure S6). In phase 2, the testing phase, when one object was replaced by a novel object, WT stressed mice failed to discriminate between the novel object and the constant object. The discrimination index (DI) of WT stressed mice (49.22 ± 2.16 , $n = 11$) was significantly lower than controls (82.44 ± 1.50 , $n = 12$, $p < 0.05$) (Figure S6). S107 treatment improved the DI of stressed mice to control level (82.59 ± 2.24 , $n = 9$, $p < 0.05$ compared to stressed WT) (Figure S6). In agreement with the MWM results, the NOR test demonstrated that chronic stress impairs memory in WT mice, and pharmacologic (S107) rescues this impairment.

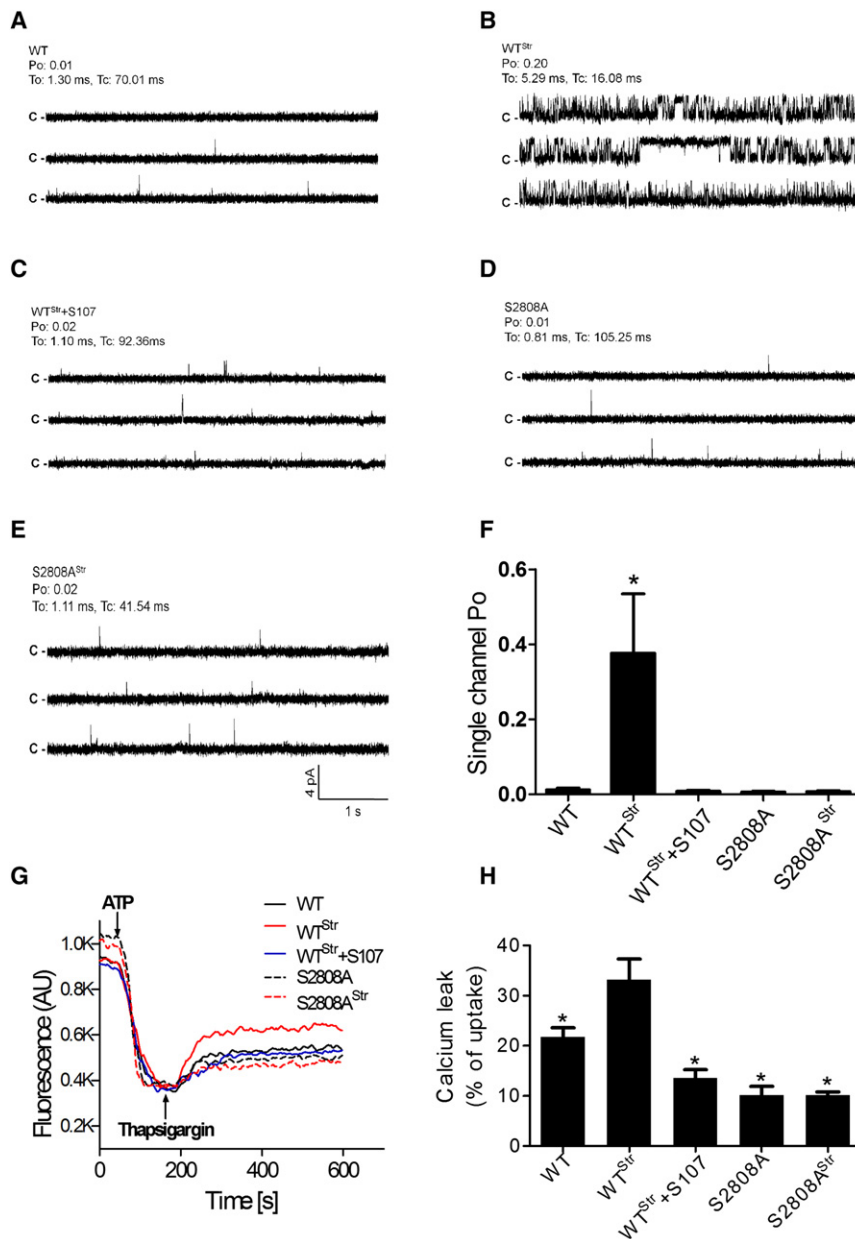


Figure 3. Single-Channel Recordings of Hippocampal RyR Channels from Stressed Mice

(A–E) Representative hippocampal RyR single-channel current traces from control (WT, $n = 6$) (A), stress (WT^{Str}, $n = 5$) (B), S107-treated stress (WT^{Str}+S107, $n = 5$) (C), RyR2-S2808A^{+/+} (S2808A, $n = 4$) (D), and stressed RyR2-S2808A^{+/+} mice (S2808A^{Str}, $n = 6$) (E) examined with 150 nmol/l (nM) free cytosolic [Ca²⁺] in the *cis* chamber. Channel openings are upward deflections, and horizontal bars at the left of each trace indicate the closed (c-) state of the channels. For each group, channel activity is illustrated with four traces, each 5 s. RyR identity was confirmed by addition of 5 μ mol/l (μ M) ryanodine at the end of each experiment. The single-channel open probability (Po), mean open time (To), and mean closed time (Tc) at 150 nmol/l free cytosolic [Ca²⁺] are above the upper trace.

(F) Summary of RyR2 single-channel Po with 150 nmol/l free cytosolic [Ca²⁺] from RyR2-WT, RyR2-WT^{Str}, WT^{Str}+S107, S2808A, and S2808A^{Str}. Data are mean \pm SEM, * $p < 0.05$ versus WT.

(G) Representative traces of Ca²⁺ leak from brain microsomes induced by addition of thapsigargin (3 μ M).

(H) Ca²⁺ leak was calculated as the percentage of uptake. Data (mean \pm SEM) analysis was performed by one-way ANOVA, $p = 0.0001$. Bonferroni posttest revealed * $p < 0.05$ versus WT^{Str} (WT, $n = 9$; WT^{Str}, $n = 7$; WT^{Str}+S107, $n = 7$; S2808A, $n = 4$; S2808A+ST, $n = 4$).

See also Figure S3H.

Elevated plus maze (EPM) (Pellow et al., 1985) and open-field (Hall, 1934) tests were used to assess anxiety and spontaneous activity in stressed WT mice. In the EPM, mice were placed at the center of the plus maze and allowed to move freely for 5 min. The time spent in the closed arm and open arm and the total number of entries to the closed arm and open arm were recorded. WT stressed mice spent significantly longer time in the closed arm (236.57 ± 9.18 s, $n = 21$) than nonstressed controls (197.86 ± 5.51 s, $n = 21$, $p < 0.05$ by two-way ANOVA) and spent significantly less time in the open arm (11.14 ± 3.24 s, $n = 21$) than controls (44.21 ± 5.0 s, $n = 21$, $p < 0.05$ by two-way ANOVA) (Figure S7). The ratio of time spent in the open arm versus closed arm was significantly reduced in WT stressed

mice (0.06 ± 0.03 , $n = 21$) compared to nonstressed controls (0.22 ± 0.05 , $n = 21$, $p < 0.05$ by two-way ANOVA) (Figure 4D). There were no significant differences in total number of entries to the closed arm among experimental groups, but there were significantly fewer entries to the open arm in WT stressed mice (1.43 ± 0.4 , $n = 21$) than in controls (4.79 ± 0.46 , $n = 21$, $p < 0.05$ by two-way ANOVA) (Figure S7). The ratio of the number of open arm entries versus closed arm entries was significantly reduced in WT stressed mice (0.23 ± 0.08 , $n = 21$) compared to nonstressed controls (0.47 ± 0.08 , $n = 21$, $p < 0.05$ by two-way ANOVA) (Figure 4E). S107 treatment significantly reduced total time spent in the closed arm (180.94 ± 4.57 s, $n = 22$, $p < 0.05$ by two-way ANOVA) but improved total time spent in the open arm (53.44 ± 4.79 s, $n = 22$, $p < 0.05$ by two-way ANOVA) in WT stressed mice (Figure S7). S107 treatment also significantly improved the ratios of time spent on the open arm versus closed arm and the number of open arm entries versus closed arm entries in stressed mice (0.29 ± 0.07 , $n = 22$, $p < 0.001$; 0.48 ± 0.06 , $n = 22$, $p < 0.001$ by two-way ANOVA) (Figure 4E). There were no differences in these two ratios when

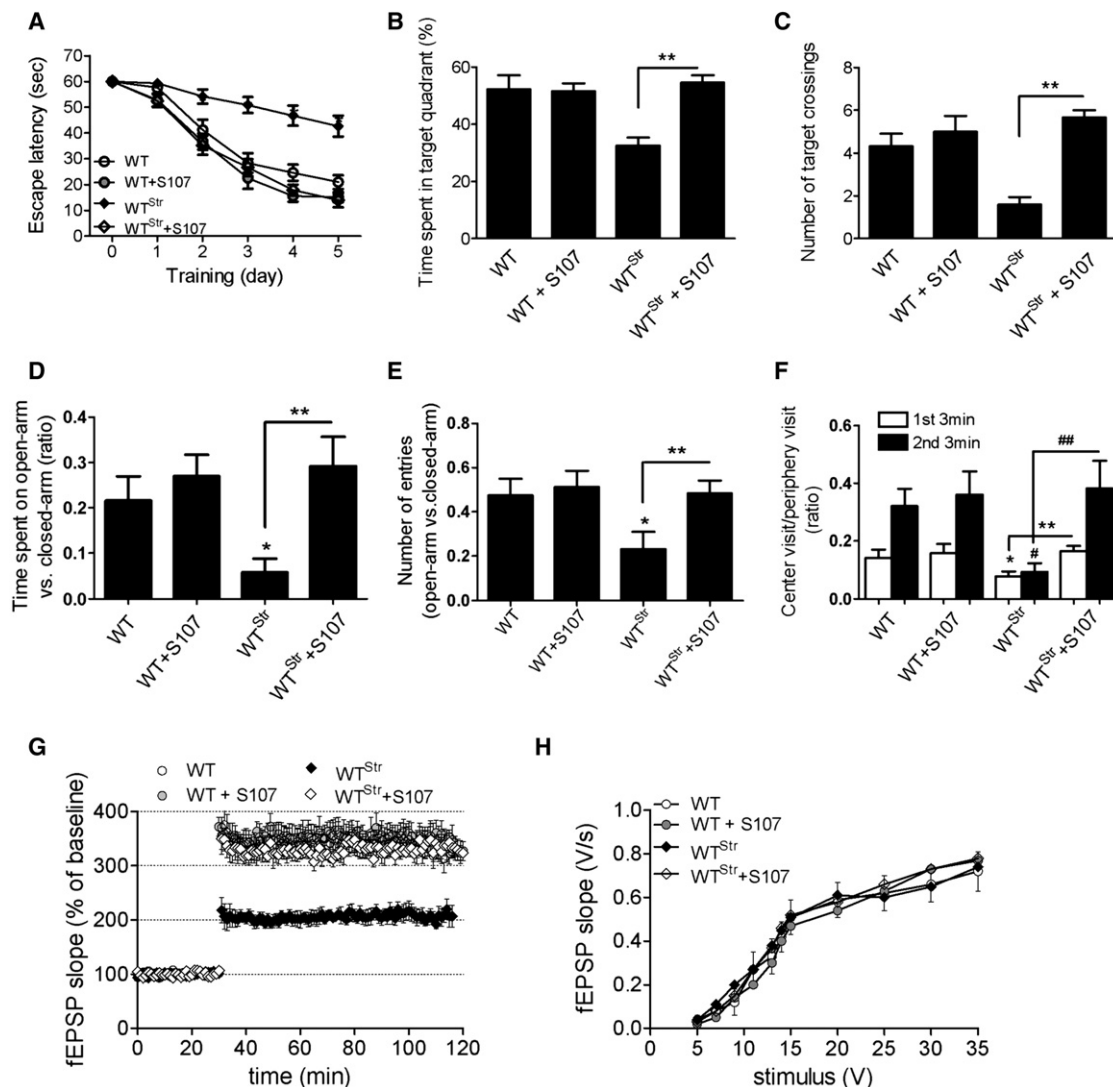


Figure 4. Effects of Stress on Cognitive Function and Hippocampal Synaptic Plasticity

(A) MWM escape latency for WT nonstressed control (WT, $n = 21$), S107-treated WT nonstressed (WT+S107, $n = 14$), WT stressed (WT^{Str}, $n = 21$), and S107-treated stressed (WT^{Str}+S107, $n = 22$).

(B and C) Probe trials measuring time spent in the target quadrant and number of target crossings. $p < 0.05$.

(D and E) EPM test on the same groups of mice. (D) Summary of ratios of time spent on the open arm versus closed arm is shown. (E) Summary data of ratios of number of entries to the open arm versus closed arm are shown.

(F) Open-field test from the same group of mice as shown in MWM and EPM tests. * $p < 0.05$ versus nonstressed controls within the first 3 min, # $p < 0.05$ versus nonstressed controls within the second 3 min.

(G) Potentiation following theta burst stimulation in the CA1 region of hippocampal slices from WT mice (WT, $n = 7$), S107-treated control (WT+S107, $n = 7$), stress (WT^{Str}, $n = 7$), and S107-treated stress (WT^{Str}+S107, $n = 7$).

(H) Summary of field input-output relationships in the same slices as in (G).

All data are mean \pm SEM. See also Figure S3G.

S107-treated nonstressed mice were compared to nontreated controls (0.27 ± 0.05 , $n = 14$, $p = 0.194$; 0.51 ± 0.07 , $n = 14$, $p = 0.24$ by two-way ANOVA) (Figures 4D and 4E), suggesting that the positive effects of S107 are not manifested in the absence of stress.

In the open-field test, mice were placed at the center of the experimental chamber and allowed to move for 6 min. The number of visits to the center area and peripheral area were

recorded and analyzed. Within the first 3 min and the second 3 min, the ratios of the number of center visits versus peripheral visits for WT stressed mice were significantly lower (0.08 ± 0.02 ; 0.09 ± 0.03 , $n = 22$) than those of nonstressed WT controls (0.14 ± 0.03 , $n = 21$, $p < 0.01$; 0.32 ± 0.06 , $p < 0.01$ by one-way ANOVA) (Figure 4F). These results are consistent with reduced spontaneous exploratory activity in the stressed mice. S107 significantly improved the exploratory activity and behavioral

response to a novel environment in stressed mice as demonstrated by the increased ratios of the number of center visits versus periphery visits within both the first 3 min and second 3 min (0.20 ± 0.04 , $p < 0.05$; 0.22 ± 0.02 , $n = 22$, $p < 0.01$ by one-way ANOVA) (Figure 4F). Taken together, the behavioral studies demonstrate that stressed mice have cognitive dysfunction manifested as impaired learning and memory, increased anxiety, and reduced spontaneous exploratory activity. Pharmacologic (S107) therapy targeting the RyR channel prevented stress-induced cognitive dysfunction, suggesting that neuronal ER Ca^{2+} leak causes cognitive dysfunction during stress.

Postsynaptic plasticity of hippocampal neurons was assessed by using electrophysiological recordings of long-term potentiation (LTP), which reflects long-term hippocampal-type memory. Theta burst stimulation (TBS) was used (12 bursts at 5 Hz, each consisting of four pulses delivered at 100 Hz) (Patterson et al., 2001) at the CA3-CA1 connection in hippocampal slices. LTP was reduced in stressed WT mice ($205.9 \pm 13.2\%$, $n = 7$) compared to nonstressed mice ($354.1 \pm 26.1\%$, $n = 7$, $p < 0.001$ by two-way ANOVA) (Figure 4G). Treatment with S107 in the drinking water during stress prevented LTP reduction ($330.4 \pm 17.6\%$, $n = 7$, $p < 0.001$) (Figure 4G). The input-output curve was not altered by either stress or S107 treatment (Figure 4H). Thus, the stress-induced impairment of hippocampal postsynaptic plasticity was significantly improved by S107.

Improved Synaptic and Cognitive Function in Stressed *RyR2-S2808A^{+/+}* Mice

RyR2-S2808A^{+/+} mice that harbor RyR2 that cannot be PKA phosphorylated were subjected to behavioral testing after 3 weeks of chronic restraint stress. In MWM tests, *RyR2-S2808A^{+/+}* mice showed the same escape latency (18.62 ± 3.05 s, $n = 13$) as WT mice (19.16 ± 3.0 s, $n = 15$, $p = 0.87$ by one-way ANOVA) (Figure 5A), indicating normal cognitive function in the *RyR2-S2808A^{+/+}* mice. The time spent in the target quadrant and the number of target crossings also showed no differences between *RyR2-S2808A^{+/+}* ($49.82 \pm 4.87\%$; 3.73 ± 0.45 crossings, $n = 13$) and WT mice ($54.4 \pm 3.62\%$, $n = 15$, $p = 0.23$; 4.13 ± 0.39 crossings, $n = 15$, $p = 0.25$) (Figures 5B and 5C). Stress failed to alter the escape latency in *RyR2-S2808A^{+/+}* mice; at day 5 of the training trial there were no significant differences in escape latency (17.43 ± 1.40 s, $n = 16$) compared to nonstressed *RyR2-S2808A^{+/+}* mice (18.62 ± 3.05 s, $n = 13$, $p = 0.85$ by one-way ANOVA) (Figure 4A). Probe trials similarly showed no deficits in stressed *RyR2-S2808A^{+/+}* mice that exhibited the same time spent in the target quadrant and number of target crossings ($50.44 \pm 2.78\%$; 4.33 ± 0.31 crossings, $n = 16$) as nonstressed *RyR2-S2808A^{+/+}* ($49.82 \pm 4.87\%$, $n = 13$, $p = 0.40$; 3.73 ± 0.45 , $n = 13$, $p = 0.14$) (Figures 5B and 5C). There were no differences in swimming velocity between groups in the MWM tests (Figure S3G).

The NOR test indicated that there was no effect of stress on memory in the *RyR2-S2808A^{+/+}* mice, as the DI in phase 2 was unchanged (Figure S6).

EPM testing showed no significant differences in total time spent in the closed arm and open arm between stressed *RyR2-S2808A^{+/+}* (161.31 ± 3.43 s in closed arm, 44.77 ± 7.55 s in open arm, $n = 16$) and nonstressed *RyR2-S2808A^{+/+}* mice

(166.21 ± 7.25 s in closed arm, $n = 15$, $p = 0.38$; 47.64 ± 6.46 s in open arm, $n = 15$, $p = 0.65$ by two-way ANOVA) (Figure S7). There were no significant differences in total number of entries to the closed arm and open arm between stressed *RyR2-S2808A^{+/+}* (9.15 ± 1.23 to the closed arm, 4.62 ± 0.77 to the open arm, $n = 16$) and nonstressed *RyR2-S2808A^{+/+}* mice (9.43 ± 1.19 to the closed arm, $n = 15$, $p = 0.80$; 4.86 ± 0.72 to the open arm, $n = 15$, $p = 0.95$ by two-way ANOVA) (Figure S7). There were no differences in time spent in the open arm versus closed arm between stressed *RyR2-S2808A^{+/+}* (0.29 ± 0.34 , $n = 16$) and nonstressed *RyR2-S2808A^{+/+}* (0.29 ± 0.31 , $n = 15$, $p = 0.92$ by two-way ANOVA) (Figure 5D). There were also no differences in the number of open-arm entries versus closed-arm entries between stressed *RyR2-S2808A^{+/+}* (0.56 ± 0.06 , $n = 16$) and nonstressed *RyR2-S2808A^{+/+}* (0.59 ± 0.10 , $n = 15$, $p = 0.79$ by two-way ANOVA) (Figure 5E), suggesting no significant changes in the level of anxiety in *RyR2-S2808A^{+/+}* mice under stress.

Open-field tests revealed that, within the first 3 min and the second 3 min, stress failed to alter the ratios of the number of center visits versus peripheral visits in *RyR2-S2808A^{+/+}* mice (0.17 ± 0.01 , $n = 16$ in stressed; 0.15 ± 0.02 , $n = 15$ in nonstressed, $p = 0.22$ by one-way ANOVA) (Figure 5F), indicating no significant stress-induced changes in spontaneous activity in the *RyR2-S2808A^{+/+}* mice.

Electrophysiological recordings revealed no differences in LTP between the *RyR2-S2808A^{+/+}* ($325.4 \pm 11.6\%$, $n = 7$) and WT mice ($329.4 \pm 18.5\%$, $n = 7$, $p = 0.88$ assessed by two-way ANOVA) (Figure 5G). Chronic stress failed to affect LTP in the *RyR2-S2808A^{+/+}* mice ($313.9 \pm 17.1\%$, $n = 7$, $p = 0.56$, assessed by two-way ANOVA) (Figure 5G). Two-way ANOVA revealed no differences in the input-output curves between WT and *RyR2-S2808A^{+/+}* mice (Figure 5H), and there were no differences in the input-output relationships between stressed and nonstressed *RyR2-S2808A^{+/+}* mice (Figure 5H).

Thus, stress-induced defects in cognitive function and postsynaptic plasticity in hippocampal neurons were ameliorated by preserving RyR2-calstabin2 interaction either by administering S107 or by genetic ablation of the RyR2 PKA phosphorylation site.

RyR1 Does Not Play a Major Role in Chronic Stress-Induced Cognitive Dysfunction

In addition to RyR2, RyR1, which is required for skeletal muscle excitation-contraction coupling, is expressed in specific regions of the brain, including the hippocampus and cerebellum (Gianini et al., 1995). We have demonstrated a role for PKA hyperphosphorylation at serine 2844 and for oxidation and nitrosylation of RyR1 in promoting SR Ca^{2+} leak in skeletal muscle during extreme exercise, muscular dystrophy, and aging (Anderson et al., 2011; Bellinger et al., 2008a, 2008b, 2009). PKA hyperphosphorylation at serine 2844 and nitrosylation and oxidation of RyR1 cause depletion of calstabin1 from the channel complex, destabilizing the closed state at low-cytosolic-activating [Ca^{2+}] (~ 150 nM) (Marx et al., 2000; Reiken et al., 2003b; Ward et al., 2003).

We subjected *RyR1-S2844A^{+/+}* knockin mice, which harbor RyR1 channels that cannot be PKA phosphorylated, to the stress

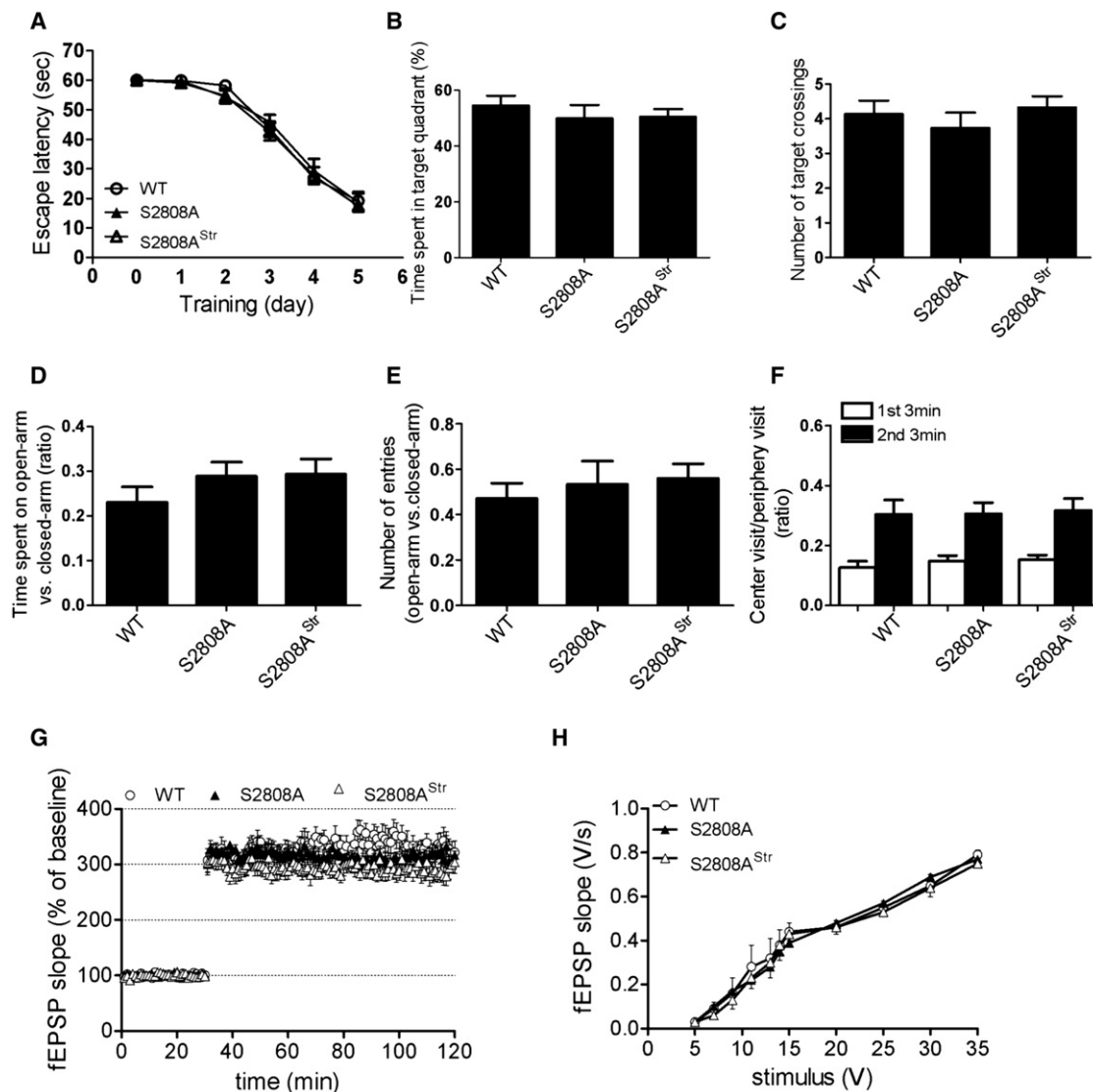


Figure 5. Effects of Stress on Cognitive Function and Hippocampal Synaptic Plasticity in *RyR2-S2808A*^{+/±} Mice

(A) MWM escape latency for WT (WT, *n* = 15), *RyR2-S2808A*^{+/±} nonstressed (S2808A, *n* = 13), and *RyR2-S2808A*^{+/±} stressed (S2808A^{Str}, *n* = 16).

(B and C) Probe trials after escape platform removed, **p* < 0.05.

(D and E) EPM test in the same groups. (D) Summary data of ratios of time spent on the open arm versus closed arm. (E) Summary data of ratios of number of entries to the open arm versus closed arm.

(F) Open-field test from the same group of mice. Ratios of total time spent in the center area versus periphery area within first 3 min and second 3 min.

(G) Potentiation following theta burst stimulation in the CA1 region of hippocampal slices from WT mice (WT, *n* = 7), *RyR2-S2808A*^{+/±} (S2808A, *n* = 7), and stressed *RyR2-S2808A*^{+/±} (S2808A^{Str}, *n* = 7).

(H) Field input-output relationships in the same slices as in (G).

See also Figures S3G, S4, S5, S6, and S7. All data are mean ± SEM.

protocol described in Figure 1A. Elevated plasma corticosterone levels (Figure 6A) and hippocampal pCREB and pERK phosphorylation confirmed the extent of stress in the *RyR1-S2844A*^{+/±} mice (Figures 6B–6D).

Neuronal RyR1 were PKA hyperphosphorylated in hippocampus (Figures 6E and 6I) and whole brain (Figures S3H–S3K) following stress and were S-nitrosylated and oxidized (Figures 6E, 6G, and 6H). There was minimal depletion of calsta-

bin1 from RyR1 (Figures 6E and 6I) in hippocampus compared to a significant reduction of calstabin1 from RyR1 in the whole brain (Figures S5E–S5G). Treatment with S107 prevented calstabin1 depletion from the RyR1 macromolecular complex in the whole brain (Figures S5E–S5G).

WT mice and *RyR1-S2844A*^{+/±} knockin mice were examined by using MWM, EPM, and open-field tasks. In the MWM task, ablation of the PKA phosphorylation site of RyR1 at serine

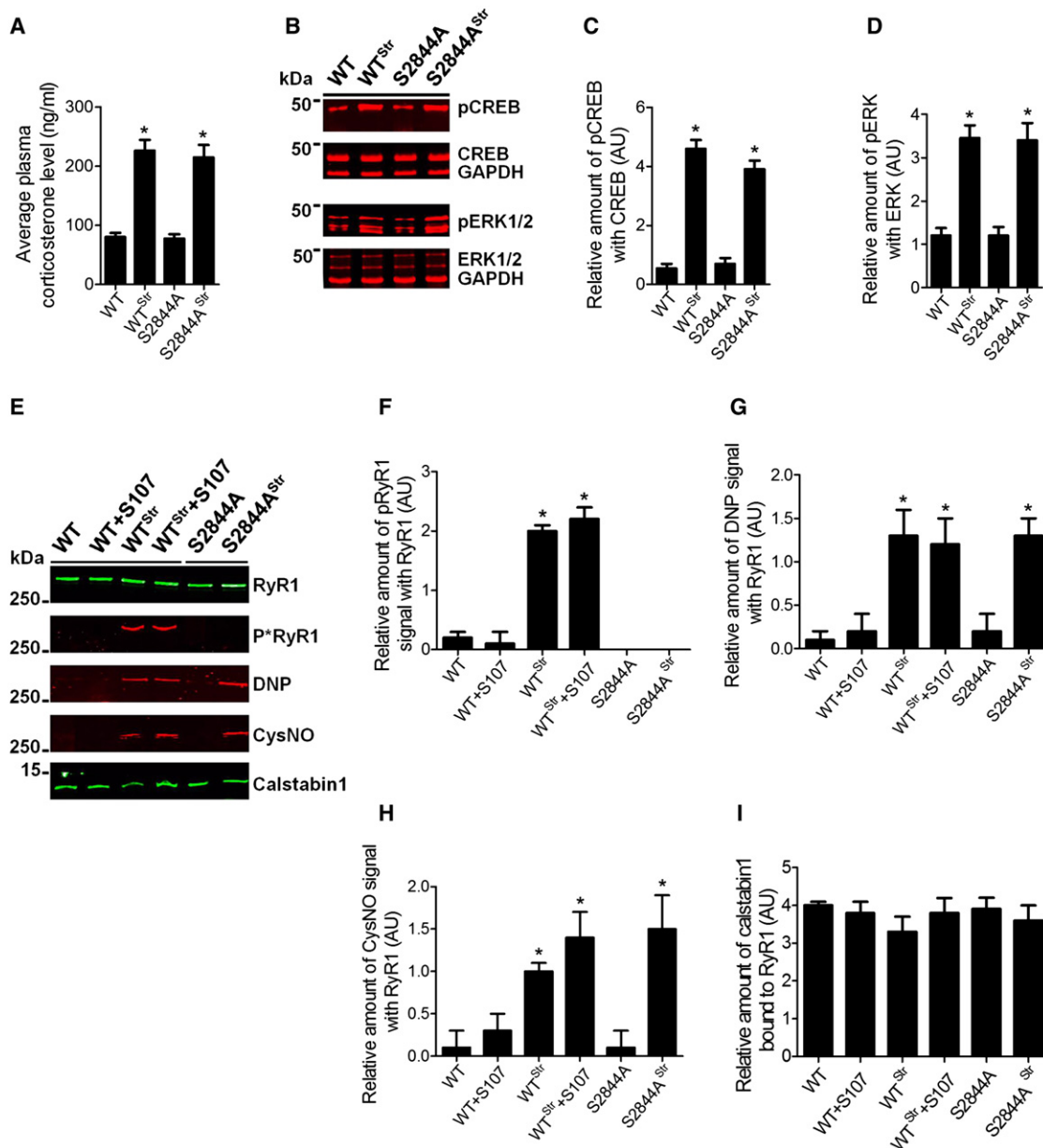


Figure 6. Stress-Induced Remodeling of RyR1 Complexes and Cognitive Function in *RyR1-S2844A*^{+/+} Mice

(A) *RyR2-S2844A*^{+/+} mice were subjected to the restraint stress protocol in Figure 1A. Morning plasma corticosterone levels collected (n = 4).

(B–D) (B) Representative immunoblots showing levels of hippocampal pCREB and pERK in control and stressed mice. Summary data for the levels of pCREB (C) and pERK (D). Total CREB, total ERK, and GAPDH were loading controls.

(E) Representative immunoblots of RyR1 immunoprecipitated from whole-brain samples from control (WT), WT stressed (WT^{Sir}), *RyR1-S2844A*^{+/+} (S2844A), and *RyR1-S2844A*^{+/+} stressed (S2844A^{Sir}) probed with antibodies showing PKA hyperphosphorylation, oxidation (DNP), Cys S-nitrosylation (CysNO) of RyR1, and coimmunoprecipitated calstabin1.

(F–I) Summary data for phosphorylated RyR1, oxidized and nitrosylated RyR1, and calstabin1. *p < 0.05, n = 4. The mean for the unstressed WT control was taken as 1.0.

All data are mean ± SEM. See also Figures S5E–S5G.

2844 did not prevent learning impairment in stressed *RyR1-S2844A*^{+/+} mice characterized by prolonged escape latency after day 3 (47.3 ± 1.9 s, n = 12, p < 0.05) compared to the non-stressed mice (37.5 ± 2.8 s, n = 30) (Figure 7A) and by less time

spent in target quadrant ($22.2 \pm 0.2\%$, n = 12, p < 0.05) and reduced target crossings (1.3 ± 0.1 , n = 12, p < 0.05) on the day 6 probe trial (Figures 7B and 7C). There were also no differences between *RyR1-S2844A*^{+/+} and WT in the escape latency

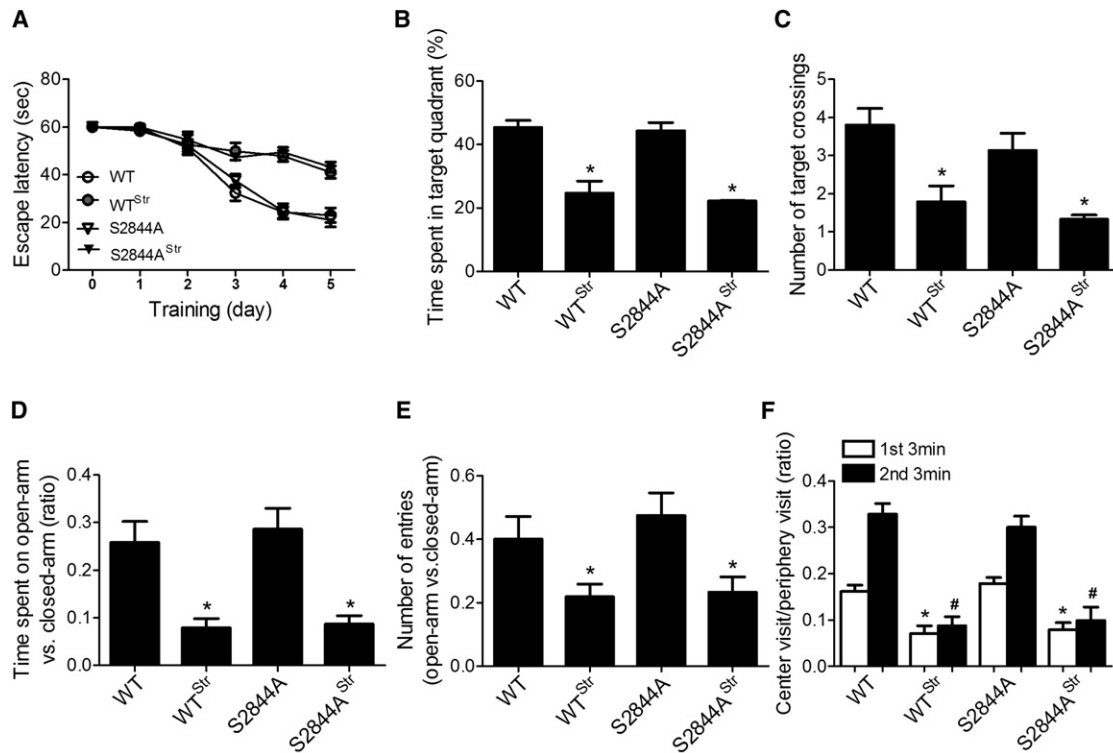


Figure 7. Effects of Stress on Cognitive Function in *RyR1-S2844A*^{+/±} Mice

(A) Mice were divided into WT (WT, *n* = 30), stressed WT (WT^{Str}, *n* = 15), *RyR1-S2844A*^{+/±} (S2844A, *n* = 30), and stressed *RyR1-S2844A*^{+/±} (S2844A^{Str}, *n* = 12). Learning curves of MWM tasks are presented as the time to reach the platform (escape latency). Mice were trained for 5 days, three trials each day, 60 s per trial. (B and C) On day 6 after 5 day training trials, the escape platform was removed, and each mouse was allowed to swim in the pool for 60 s. Total time spent in the target quadrant and the number of target crossings were recorded. The escape latency and the ratios of swimming time spent in the target quadrant were recorded and analyzed as mean ± SEM, **p* < 0.05. The results were analyzed using one-way ANOVA and comparison *t* test.

(D and E) Results of EPM test from the same groups of mice as shown in MWM test. (D) Summary data of ratios of time spent on the open arm versus closed arm are shown. (E) Summary data of ratios of number of entries to the open arm versus closed arm are shown. The results of EPM were analyzed using two-way ANOVA and comparison *t* test.

(F) Results of open-field test from the same group of mice as shown in MWM and EPM tests. Ratios of total time spent in the center area versus periphery area within first 3 min and second 3 min are shown. Data were analyzed by one-way ANOVA and comparison *t* test.

observed during the 5 day trial (*p* = 0.95 by one-way ANOVA) (Figure 7A). The time spent in the target quadrant and the number of target crossings were not significantly different between WT (45.3 ± 2.3%; 3.8 ± 0.4, *n* = 30) and nonstressed *RyR1-S2844A*^{+/±} mice (44.3 ± 2.7%, *n* = 30, *p* = 0.38; 3.1 ± 0.5, *n* = 30, *p* = 0.15) (Figures 7B and 7C). No differences in swimming velocity among the groups were observed (Figure S3G).

EPM tests showed no significant differences in time spent in the open arm versus the closed arm between *RyR1-S2844A*^{+/±} (0.29 ± 0.04, *n* = 30) and WT mice (0.26 ± 0.04, *n* = 30, *p* = 0.66 by two-way ANOVA) (Figure 7D). There were also no differences in the number of open-arm entries versus closed-arm entries between *RyR1-S2844A*^{+/±} (0.47 ± 0.07, *n* = 30) and WT (0.40 ± 0.07, *n* = 30, *p* = 0.55 by two-way ANOVA) (Figure 7E). Ablation of the RyR1 PKA phosphorylation site at serine 2844 failed to prevent the increase in anxiety in the stressed *RyR1-S2844A*^{+/±} mice. The time spent on the open arm versus closed arm and the number of entries to the open arm versus closed arm in stressed *RyR1-S2844A*^{+/±} mice were significantly reduced (0.08 ± 0.02, *n* = 12, *p* < 0.05; 0.29 ± 0.05, *n* = 12, *p* < 0.05

by two-way ANOVA) compared to the nonstressed *RyR1-S2844A*^{+/±} mice (Figure 7E). Open-field tests from the same group of animals as in the MWM and EPM revealed that, within the first 3 min and the second 3 min, the ratios of the number of center visits versus peripheral visits in stressed *RyR1-S2844A*^{+/±} mice were significantly lower (0.11 ± 0.02; 0.12 ± 0.02, *n* = 12) than those of nonstressed *RyR1-S2844A*^{+/±} (0.17 ± 0.02, *n* = 30, *p* < 0.05; 0.32 ± 0.03, *p* < 0.05 by one-way ANOVA) (Figure 7F). There were no differences between the *RyR1-S2844A*^{+/±} and WT mice in the open-field tests (*p* = 0.46, *p* = 0.79 by one-way ANOVA) (Figure 7F). Thus, unlike RyR2, remodeling of the RyR1 macromolecular complex does not appear to play a role in stress-induced cognitive dysfunction.

DISCUSSION

There is a well-established link between impaired learning, working, and long-term memory and somatic factors, including mental stress and chronic diseases (Etgen et al., 2009). Stress affects the function and morphology of neurons (Kim and

Diamond, 2002) and hippocampal neurogenesis (Mirescu and Gould, 2006). Moreover, it has been proposed that these effects could underlie neuropsychiatric disorders and PTSD (Airan et al., 2007; Mirescu and Gould, 2006). Long-term, chronic stress is a major contributor to the development of neuropsychiatric, cardiovascular, and autoimmune diseases, as well as cancer and propensity to self-administer drugs of abuse (McEwen, 2008). The stress response is characterized by a rapid increase in norepinephrine (NE) and epinephrine (Epi) to maintain systemic “homeostasis” (Cannon, 1929; McMahon et al., 1992; Tsuda and Tanaka, 1985; Zigmond et al., 1989). Increased sympathetic activation and urinary NE and/or Epi concentrations are consistently observed in patients with PTSD (Southwick et al., 1999; Yehuda et al., 1998), suggesting long-term chronic stress (Miller and McEwen, 2006; Sherin and Nemeroff, 2011). Despite all of these parallels between chronic stress and PTSD, one potential caveat is that the chronic restraint stress model used in the present study likely does not recapitulate all of the parameters of PTSD.

Current therapy for PTSD remains largely supportive and not mechanism based. Here, we examined the potential role of stress-induced intracellular Ca^{2+} leak due to stress-induced remodeling of RyR/calcium release channels that have not previously been implicated in stress-induced cognitive dysfunction. PKA phosphorylation of RyR1 and RyR2 (Marx et al., 2000; Reiken et al., 2003a, 2003b) at serine 2844 or 2808, respectively, increases channel activity and intracellular Ca^{2+} release during acute stress (e.g., exercise) (Marx et al., 2000; Reiken et al., 2003b). RyR dysfunction and pathologic intracellular Ca^{2+} leak occur during intense chronic β -adrenergic stimulation due to sympathetic hyperactivity (Marx et al., 2000). We have developed an RyR-targeted small molecule Rycal (S107) that stabilizes the RyR1 and RyR2 closed states of PKA-hyperphosphorylated and oxidized/nitrosylated channels and prevents intracellular Ca^{2+} -leak-induced pathophysiology in relevant animal models of heart failure, exercise-induced arrhythmias, muscular dystrophies, and sarcopenia (Andersson et al., 2011; Beltinger et al., 2008b, 2009; Lehnart et al., 2006; Wehrens et al., 2004, 2005).

Elevated intracellular Ca^{2+} has been associated with cognitive dysfunction due to brain injury (Deshpande et al., 2008) and neurodegeneration, including Alzheimer's disease and aging (Berridge, 2010; Keefe et al., 1989; Khachaturian, 1994). One of the downstream consequences of increased RyR2-mediated Ca^{2+} leak could be abnormal action potential conduction in hippocampal pyramidal neurons (Svoboda et al., 1997). Neuronal cell death could also be a consequence of Ca^{2+} leak because sustained increases in cytosolic Ca^{2+} resulting from excessive glutamate mediate excitotoxic cell death (Choi, 1988a, 1988b; Hartley et al., 1993). Other downstream targets include Ca^{2+} -dependent proteases and transcriptional activation through CaMKII-, PKA-, and MAPK-mediated activation of immediate early genes (Ghosh and Greenberg, 1995).

Intracellular Ca^{2+} homeostasis is also regulated by the IP3Rs and SERCA pumps on the ER (Berridge et al., 2003). Studies have shown improved cognitive function in mice due to blockade of IP3R in prefrontal cortex under stress conditions (Brennan et al., 2008). Another study showed increased IP3R-mediated

ER Ca^{2+} release in hippocampal neurons during hypoxia (Bickler et al., 2009). We did not observe stress-induced effects on IP3R1 (Figure S5H) or SERCA (Figure S3F) in contrast to the stress-induced remodeling of RyR2 that rendered the channels leaky. Although RyR1 and RyR2 are structurally and functionally homologous, the fact that *RyR1-S2844A*^{+/+} mice did not protect against stress-induced cognitive dysfunction, whereas *RyR2-S2808A*^{+/+} did, suggests that RyR2, but not RyR1, is involved in stress-induced cognitive dysfunction.

Thus, neuronal RyR2 remodeling by PKA hyperphosphorylation, oxidation, and nitrosylation plays an important role in chronic stress-induced cognitive dysfunction. Furthermore, leaky neuronal RyR2 channels could be a potential pharmacological target to ameliorate chronic stress-induced cognitive dysfunction and PTSD.

EXPERIMENTAL PROCEDURES

Animals and Restraint Stress Protocol

WT C57BL/6 mice and age-matched *RyR2-S2808A*^{+/+} and *RyR1-S2844A*^{+/+} knockin mice (Wehrens et al., 2005) were subjected to restraint stress in 50 ml plastic restraint tubes (Plas-Labs Broome-Style Rodent Restraint, from Fisher Scientific) without food and water from 6 PM to 9 AM for 3 weeks (Copeland et al., 2005). Schedules for applying restraint stress and performing cognition function tests are illustrated in Figure 1A. Investigators were blinded with respect to genotype and treatment.

Statistical Analysis

MWM and open-field tests were analyzed by using one-way ANOVA and comparison t test. EPM and LTP measurements were analyzed by using two-way ANOVA and comparison t test. All other data were expressed as mean \pm SEM. The results of Ca^{2+} leak assay were analyzed by one-way ANOVA with Bonferroni correction. Minimum statistically significant differences were established at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2012.06.052>.

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